

Identification of the polypeptides encoded in the ATPase 6 gene and in the unassigned reading frames 1 and 3 of human mtDNA

(HeLa cells/synthetic peptides/antibodies/mitochondrial translation products)

ANNE CHOMYN*, PAOLO MARIOTTINI*, NESTOR GONZALEZ-CADAVID*†, GIUSEPPE ATTARDI*,
DONNA D. STRONG‡, DENNIS TROVATO‡, MARCIA RILEY‡, AND RUSSELL F. DOOLITTLE‡

*Division of Biology, California Institute of Technology, Pasadena, California 91125; and †Department of Chemistry, University of California at San Diego, La Jolla, California 92093

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ABSTRACT Antibodies prepared against chemically synthesized peptides predicted from the DNA sequence have been used to identify the polypeptides encoded in the ATPase 6 gene and in unidentified reading frames (URFs) 1 and 3 of human mtDNA. In particular, antibodies directed against the COOH-terminal nonapeptide of the putative polypeptide encoded in the ATPase 6 reading frame immunoprecipitated specifically component 17 of the HeLa cell mitochondrial translation products, the reaction being inhibited by the specific peptide. Similarly, antibodies directed against the COOH-terminal undecapeptide of the putative URF1 product or against the COOH-terminal heptapeptide of the presumptive URF3 product were effective in immunoprecipitating specifically component 12 or, respectively, component 24 of the mitochondrial translation products. The sizes of proteins 17, 12, and 24, as estimated from their electrophoretic mobilities, are compatible with their being the products of the ATPase 6 gene, URF1, and URF3, respectively.

The recent sequence analysis of human mtDNA (1) has revealed that this genome has an informational content larger than previously suspected. In fact, besides five structural genes homologous to known genes of yeast and *Neurospora crassa* mtDNA, eight significant reading frames (>200 nucleotides) have been found (Fig. 1). These reading frames are perfectly conserved in their size and mapping position in mouse (5), bovine (6), and rat (7, 8) mtDNAs. With the aims of determining whether these unidentified reading frames (URFs) are expressed in human mitochondria and of identifying their protein products among the mitochondrially synthesized polypeptides (9), we have taken advantage of a recently developed approach (for review, see ref. 10) and used antibodies directed against synthetic peptides corresponding to specific regions of the URFs (11). By using this approach, we have identified (11) the product of the smallest among the human URFs, URFA6L, a 207-nucleotide-long reading frame overlapping out of phase the ATPase 6 gene (Fig. 1), as corresponding to component 25 of the mitochondrial translation products in HeLa cells (9).

Only one mRNA, RNA 14 (12), has been found to map in the region of URFA6L and of the ATPase 6 gene of human mtDNA (2). Because of the interesting questions that the unusual organization of these two genes raise concerning the translation of their products, it seemed important to show that the ATPase 6 gene is expressed in HeLa cells. In the present work, the use of antibodies directed against a synthetic peptide predicted from the DNA sequence has led to the identification, among the mitochondrially synthesized polypeptides of HeLa cells, of component 17 as the product of the ATPase 6 gene. By the same

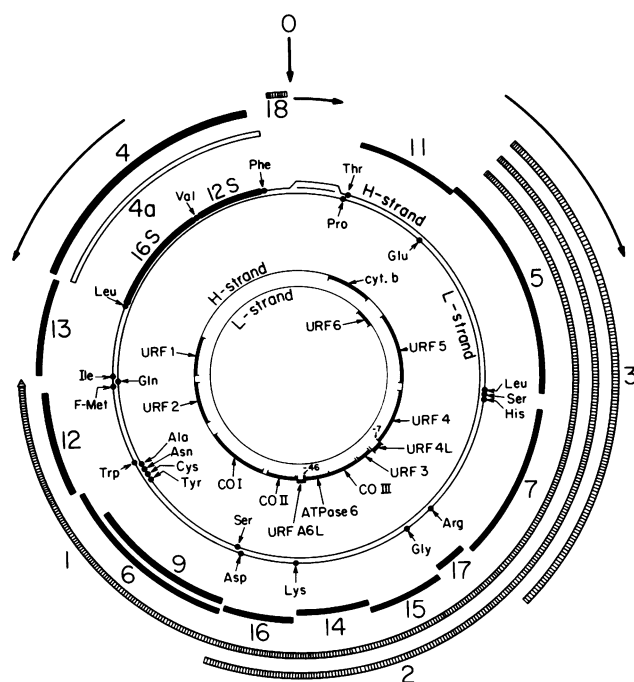


FIG. 1. Genetic and transcriptional maps of the HeLa cell mitochondrial genome (1, 2). The two outer circles show the positions of the two rRNA genes, as derived from mapping and RNA sequence analysis experiments (2, 3), and those of the tRNA genes, as derived from the mtDNA sequence (1). Mapping positions (2) of the polyadenylated H-strand transcripts are indicated by black bars and those of the L-strand transcripts, by hatched bars. Left and right arrows indicate the direction of H- and L-strand transcription, respectively; the vertical arrow (marked 0) and the rightward arrow at the top indicate the location of the origin and the direction of H-strand synthesis. The two inner circles show the positions of the mtDNA reading frames (1). Modified from ref. 4.

approach, components 12 and 24 (9) have been identified as the products of URF1 and URF3, respectively.

MATERIALS AND METHODS

Peptide Synthesis and Attachment to Carrier Proteins and Preparation of Antisera. The techniques used for the synthesis and characterization of the peptides, their attachment to bovine serum albumin or horse myoglobin, and the preparation and testing of antisera have been described (11, 13).

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Abbreviation: URF, unidentified reading frame.

† Present address: Departamento de Biología Celular, Universidad Central de Venezuela, Caracas, Venezuela.

Preparation and Testing of Gamma Globulin and Removal of Anti-Bovine Serum Albumin Antibodies. A gamma globulin fraction was obtained from the antisera and from one normal rabbit serum by a single precipitation with 33% $(\text{NH}_4)_2\text{SO}_4$ and freed of anti-bovine serum albumin antibodies by two consecutive passages through a column of bovine serum albumin conjugated to Sepharose 4B (Pharmacia). The immunoreactivity of the final gamma globulin preparations against the synthetic peptides was tested by a solid-phase radioimmunoassay (11).

Labeling and Immunoprecipitation of Mitochondrial Translation Products and Analysis of Immunoprecipitate. HeLa cells were labeled with [^{35}S]methionine in the presence of emetine (9), and the mitochondrial fraction was isolated and sonicated as described (11). Immunoprecipitation of the products and electrophoretic analysis of the immunoprecipitate was carried out as detailed (11). In some experiments, the immunoprecipitate was electrophoresed through a linear gradient polyacrylamide gel (9).

Analysis of Crossreactivity of Antipeptide Antibodies by Solid-Phase Radioimmunoassay. This was carried out by the previously described radioimmunoassay (11), modified as described below. A solution (1 mg/ml) of the particular myoglobin-peptide conjugate (the degree of substitution was 26 peptides URFA6L-C, 16 peptides URF1-C, 20 peptides ATPase 6-C per myoglobin molecule), or of horse myoglobin, or of human fibrinogen was deposited in each well of a microtiter plate, and the wells were washed twice with phosphate-buffered saline containing ovalbumin (4 mg/ml), with the last solution being left for 2 hr at room temperature. Then, the antiserum at various dilutions was deposited in the wells and left for 1 hr at 37°C. After this, the wells were washed three times with 0.05% Nonidet P-40 in phosphate-buffered saline, and 125,000 cpm of [^{125}I]-labeled goat anti-rabbit gamma globulin ($5\text{--}10 \times 10^5$ cpm/ μg) was deposited in each well and left for 1 hr at 37°C. Finally, the wells were washed three times with Nonidet P-40/phosphate-buffered saline, their bottoms were cut out, and radioactivity was determined in a gamma counter.

RESULTS

Preparation of Antipeptide Antibodies. In the present work, a COOH-terminal sequence of the putative products of the ATPase 6 gene and of URF1 and two COOH-terminal sequences of different lengths of URF3 were chosen for chemical synthesis. Table 1 shows the sequences of the peptides synthesized, their mode of attachment to bovine serum albumin, and the degree of substitution obtained. The preparation of antibodies against a COOH-terminal undecapeptide of cytochrome *c* oxidase subunit II (COII) (anti-COII-C) has been described (11).

Immunoprecipitation of Mitochondrial Translation Products with Anti-ATPase 6-C Antibodies. Immunoprecipitation tests were carried out using a HeLa cell mitochondrial lysate,

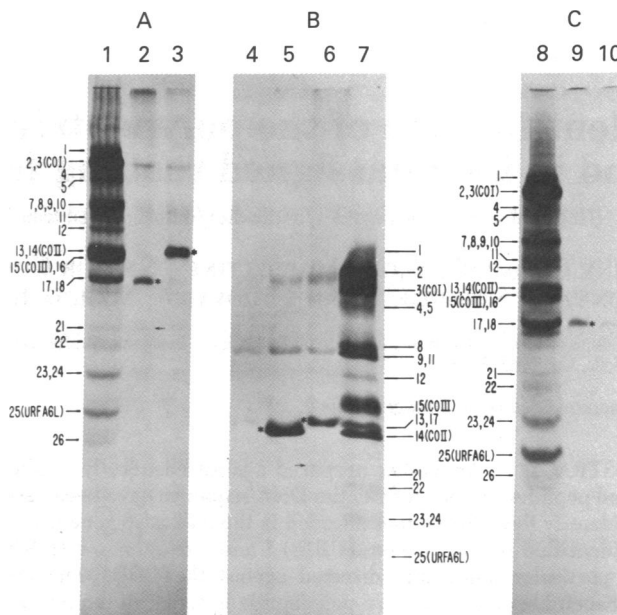


Fig. 2. Fluorograms, after electrophoresis through NaDodSO₄/urea/polyacrylamide gels (A and C) or a NaDodSO₄/gradient polyacrylamide gel (B), of the HeLa cell mitochondrial translation products immunoprecipitated by anti-ATPase 6-C or anti-COII-C antibodies. (A and B) Immunoprecipitation tests using [^{35}S]methionine-labeled mitochondrial translation products and 1 mg of gamma globulin from an anti-ATPase 6-C (lanes 2 and 6) or an anti-COII-C (lanes 3 and 5) antiserum, freed of anti-bovine serum albumin antibodies. A control experiment using 1 mg of gamma globulin from a normal rabbit serum is shown in lane 4. (C) Immunoprecipitation tests using 1 mg of gamma globulin from an anti-ATPase 6-C antiserum in the absence (lane 9) or presence (lane 10) of 20 μg of ATPase 6-C peptide. Lanes 1, 7, and 8: pattern of mitochondrial translation products.

labeled with [^{35}S]methionine under conditions selective for mitochondrial protein synthesis, and gamma globulin from an antiserum directed against the COOH-terminal peptide of the putative ATPase 6 gene product (anti-ATPase 6-C), freed of anti-bovine serum albumin antibodies. After NaDodSO₄/urea/polyacrylamide gel electrophoresis of the immunoprecipitated material and fluorography, a distinct band with a mobility corresponding to that of the band designated "17,18" in the profile of HeLa cell mitochondrial translation products (9) was observed (Fig. 2A). Only a barely visible band, representing non-specific background, was present at the same position in the immunoprecipitate obtained with anti-COII-C gamma globulin.

Previous work (9) had shown that the two components forming band "17,18" can be resolved in a different gel system—i.e., a linear gradient polyacrylamide slab gel, with component 17, which is the major one, migrating slower than COII [rather than

Table 1. Synthetic peptides and modes of attachment

Source	Sequence [†]	Attachment procedure	Degree of substitution [‡]
ATPase 6 (carboxyl terminus)	(Lys)Val [*] -Ser-Leu-Tyr-Leu-His-Asp-Asn-Thr (<i>n</i> = 10)	Glutaraldehyde	21
URF1 (carboxyl terminus)	(Lys)Pro [*] -Ile-Thr-Ile-Ser-Ser-Ile-Pro-Pro-Gln-Thr (<i>n</i> = 12)	Glutaraldehyde	26
URF3 (carboxyl terminus) 1	Lys-Gly [*] -Leu-Asp-Trp-Thr-Glu	Glutaraldehyde	35
2	(Tyr)Leu-Gln-Lys-Gly [*] -Leu-Asp-Trp-Thr-Glu	Bisdiazobenzidine	28

[†]Residues in parentheses were incorporated for attachment purposes. *, Radioactive residue.

[‡]Number of peptide molecules per bovine serum albumin molecule.

faster, as in the urea gel system (Fig. 2A)], and component 18 moving faster than COII. As shown in Fig. 2B, in this gradient gel system, a pronounced band with mobility corresponding to that of component 17 was visible in the pattern of the products precipitated by anti-ATPase 6-C antibodies, while the COII polypeptide precipitated by anti-COII antibodies formed a band at the expected position. Fig. 2C shows that the ATPase 6 COOH-terminal peptide (added in the amount of 20 μ g) competed almost completely ($\approx 80\%$) with component 17 in the precipitation by the antipeptide antibodies (the competition was essentially complete with a larger amount of peptide), confirming the specificity of the reaction.

In the electrophoretic pattern of the immunoprecipitate obtained with anti-ATPase 6-C gamma globulin, a very faint band, absent in the control lanes, could be seen after long exposure of the autoradiogram (arrow in Fig. 2A and B). In both the urea gel pattern (Fig. 2A) and the gradient polyacrylamide gel pattern (Fig. 2B), this band had a mobility similar to that of component 21. It seems possible that this peptide represents a COOH-terminal degradation fragment of ATPase 6.

Immunoprecipitation of Mitochondrial Translation Products with Anti-URF1-C Antibodies. Fig. 3A shows the electrophoretic pattern in a NaDodSO₄/urea/polyacrylamide gel of the [³⁵S]methionine-labeled components immunoprecipitated by anti-URF1-C gamma globulin. One can see a pronounced band with a mobility corresponding to that of component 12 and a less pronounced band migrating as protein 25, which are absent in the immunoprecipitate obtained with gamma globulin from an anti-URF3-C antiserum. After electrophoresis of the immunoprecipitated components through a NaDodSO₄/gradient polyacrylamide gel (Fig. 3B), one can see likewise a pronounced band with a mobility identical to that of component 12 and a less pronounced band migrating as protein 25, which are absent in the control immunoprecipitate obtained with normal serum gamma globulin.

In previous work (11), it had been observed that anti-URFA6L-

C antibodies, but not anti-URFA6L-N antibodies, precipitated component 12, although with a much lower efficiency (10–20%), as compared with protein 25. This crossreactivity of component 12 and protein 25 with anti-URFA6L-C antibodies had been interpreted as suggesting that protein 12 is the product of URF1; in fact, this is the only one among the potential human mtDNA gene products deduced from the DNA sequence that shows significant sequence homology (occurring in its COOH-terminal nonapeptide) to the COOH-terminal nonapeptide of protein 25 (see Discussion). The present results have corroborated the above interpretation by revealing that there is also a crossreactivity of component 12 and protein 25 with the anti-URF1-C antibodies. From densitometric measurements, one can estimate that the efficiency of immunoprecipitation of component 25 by anti-URF1-C antibodies was about 60% of that observed for component 12. Fig. 3D shows that 20 μ g of the COOH-terminal peptide of the URF1 product competed completely with both protein 12 and protein 25 in the precipitation by antipeptide antibodies.

Quantitation of the Crossreactivity of the URF1-C and URFA6L-C Peptides with Each of the Corresponding Antisera. To quantitate the crossreactivity of the two specific peptides with the anti-URF1-C or anti-URFA6L-C antibodies, without interference by the rest of the molecule or by the NaDodSO₄ present in the immunoprecipitation mixture, solid-phase radioimmunoassays were carried out using myoglobin-URF1-C peptide and myoglobin-URFA6L-C peptide conjugates as antigens, anti-URF1-C and anti-URFA6L-C antisera, and ¹²⁵I-labeled goat anti-rabbit gamma globulin. The URF1-C and URFA6L-C peptides appeared to crossreact almost completely with the anti-URF1-C antiserum, with a slightly stronger reaction of the antiserum with the URF1-C peptide at the higher dilutions (Fig. 4a). By contrast, the URF1-C peptide crossreacted incompletely with the URFA6L-C peptide vs. the anti-URFA6L-C antiserum, especially in the higher dilution range (60–70%) (Fig. 4b). No reaction with myoglobin or the myoglo-

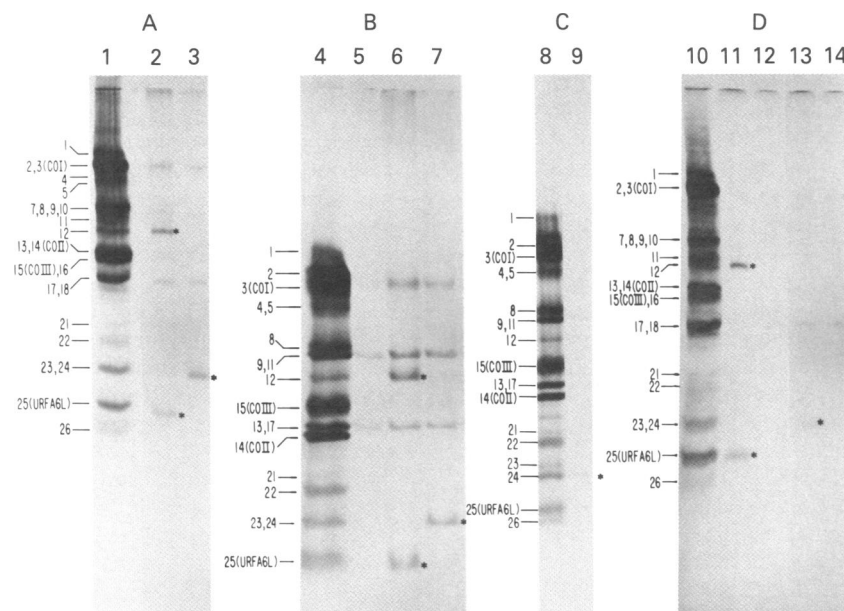


FIG. 3. Fluorograms, after electrophoresis through NaDodSO₄/urea/polyacrylamide gels (A and D) or NaDodSO₄/gradient polyacrylamide gels (B and C) of the HeLa cell mitochondrial translation products immunoprecipitated by anti-URF1-C or anti-URF3-C antibodies. (A–C) Immunoprecipitation tests using [³⁵S]methionine-labeled mitochondrial translation products and 1 mg of gamma globulin from an anti-URF1-C (lanes 2 and 6) or an anti-URF3-C (lanes 3, 7 and 9) antiserum, freed of anti-bovine serum albumin antibodies. A control experiment using 1 mg of gamma globulin from a normal rabbit serum is also shown (lane 5). (D) Immunoprecipitation tests using 1 mg of gamma globulin from an anti-URF1-C antiserum in the absence (lane 11) or presence (lane 12) of 20 μ g of URF1-C peptide or using 1 mg of gamma globulin from an anti-URF3-C1 antiserum in the absence (lane 13) or presence (lane 14) of 20 μ g of URF3-C1 peptide. Lanes 1, 4, 8 and 10: pattern of mitochondrial translation products.

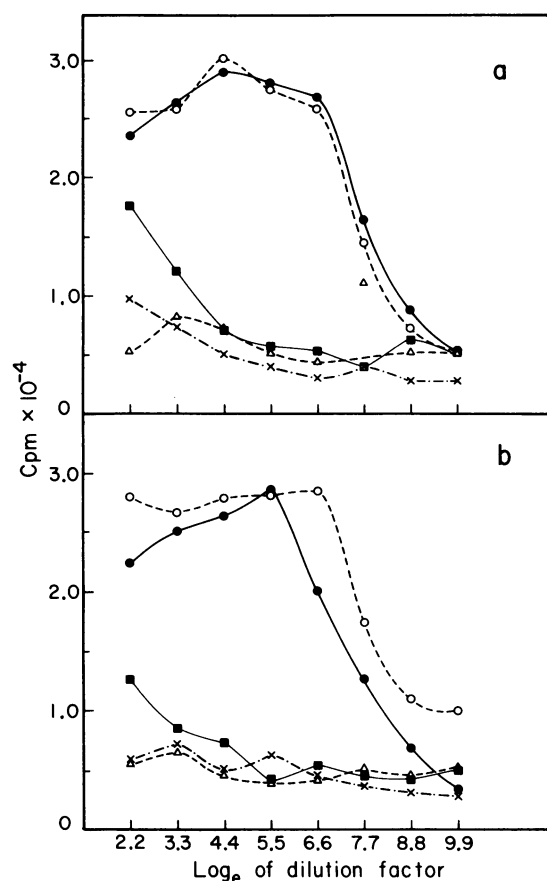


FIG. 4. Reactivity of anti-URF1-C antiserum (a) or anti-URFA6L-C antiserum (b) with myoglobin-URF1-C conjugate (●), myoglobin-URFA6L-C conjugate (○), horse myoglobin (×), myoglobin-ATPase 6-C conjugate (▲), or human fibrinogen (■).

bin-ATPase 6-C peptide was exhibited by either the anti-URF1-C or the anti-URFA6L-C antiserum.

Immunoprecipitation of Mitochondrial Translation Products with Anti-URF3-C Antibodies. The initial tests by solid-phase radioimmunoassay (11) showed that the anti-URF3-C1 antiserum reacted equally well with URF3-C1 and URF3-C2 (Table 1); the same was true for anti-URF3-C2 antiserum. However, surprisingly, the anti-URF3-C1 antiserum was about 10 times more active than the anti-URF3-C2 antiserum; by contrast, the two antisera were similarly active when tested against bovine serum albumin. This result suggested that the COOH-terminal nonapeptide was much less immunogenic than the COOH-terminal heptapeptide. Therefore, the anti-URF3-C1 antiserum was used in subsequent experiments.

Analysis in a NaDodSO₄/urea/polyacrylamide gel of the [³⁵S]methionine-labeled material immunoprecipitated by anti-URF3-C1 gamma globulin revealed a distinct band, with a mobility corresponding to that of the band designated "23,24" in the profile of HeLa cell mitochondrial translation products (9) (Fig. 3A); only a very faint band is visible at the same position in the immunoprecipitate obtained with anti-URF1-C gamma globulin. In a NaDodSO₄/linear gradient polyacrylamide slab gel, the component precipitated by anti-URF3-C1 gamma globulin again migrated with the same mobility as the unresolved components "23,24" (Fig. 3B). In an electrophoretic run in the same gel system in which components 23 and 24 were resolved, the immunoprecipitated component could be identified as component 24 (Fig. 3C). Fig. 3D shows that 20 μg of the COOH-terminal peptide of the URF3 product competed completely

with protein 24 in the precipitation by the antipeptide antibodies.

DISCUSSION

Identity of the Polypeptides Precipitated by the Antipeptide Antibodies. Component 17 of the HeLa cell mitochondrial translation products, the polypeptide specifically precipitated in this work by anti-ATPase 6-C antibodies, has been estimated to have a size of ≈16,000 daltons on the basis of its electrophoretic mobility in a NaDodSO₄/urea/polyacrylamide gel (9). This size is smaller by 33% than that expected for the protein encoded in the ATPase 6 reading frame [24,000 daltons (1)]. However, it has been shown that NaDodSO₄/polyacrylamide gel electrophoresis has a tendency to give underestimates, by 20–40%, of the size of the mtDNA-encoded hydrophobic proteins relative to the size expected from the DNA sequence (1, 14–17). Furthermore, in the case of component 17, a processing step at the NH₂ terminus could conceivably contribute to the apparent difference in size from the expected one.

The same bias in the size estimates based on NaDodSO₄/polyacrylamide gel electrophoresis data is probably responsible in major part, if not completely, for the discrepancies between the expected size of the URF1 product [35,600 daltons (1)] and the estimated size of protein 12, precipitated by anti-URF1 antibodies [≈24,000 daltons (9)], and between the expected size of the URF3 product [13,200 daltons (1)] and the estimated size of the immunoprecipitated component 24 [≈6,000 daltons (9)]. It should also be noted that, apart from a possible processing, secondary modifications of the primary translation products could also affect their electrophoretic mobilities in a NaDodSO₄/urea/polyacrylamide gel.

The specificity of the individual immunoprecipitation reactions and the general agreement of the sizes of the immunoprecipitated components with the expected ones support the conclusion that the polypeptides recognized by the antipeptide antibodies are the true gene products of the ATPase 6 gene and of URF1 and URF3. In agreement with previous experience (10, 11), the COOH-terminal peptide of the products of the reading frames investigated here has proven in all cases to be both immunogenic and capable of reacting, in the intact protein, with the antipeptide antibodies under the conditions chosen for immunoprecipitation. Unexpected, however, was the observation that the COOH-terminal nonapeptide of URF3-C was much less immunogenic than the COOH-terminal heptapeptide.

Mechanism of Expression of the ATPase 6 Gene. The evidence obtained in this and in previous work (11) that the ATPase 6 gene and URFA6L are both expressed in human mitochondria raises intriguing questions concerning the mechanism whereby the products of these two overlapping genes are synthesized. A single H-strand-encoded RNA, polyadenylated RNA 14 (12), has been found to map in this region of human mtDNA. This RNA has the unique structural features at the 5' end and at the 3' end and the characteristic metabolic properties that have been described for human mitochondrial mRNAs (18–20). Moreover, on the basis of the available evidence, it seems likely that the same RNA is used not only for the translation of URFA6L, which is not surprising in view of its typical 5'-end structure, but also for translation of the ATPase 6 gene.

In prokaryotic and eukaryotic systems, several examples of overlapping reading frames are known, and various mechanisms have been proposed to account for their expression (21–24). In the case of the overlapping URFA6L and ATPase 6 coding sequences, the lack of a ribosome attachment site, analogous to that present in 7S RNA (25), in the segment of RNA 14

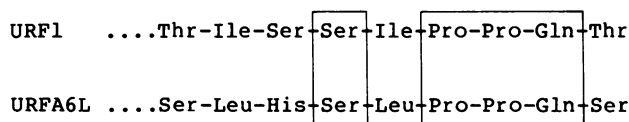


FIG. 5. Carboxyl-terminal nonapeptides of the putative products of URF1 and URFA6L.

upstream of the ATPase 6 reading frame speaks against an independent entry site for ribosomes destined to translate the downstream reading frame. A possible mechanism operating in the translation of the ATPase 6 gene is a frameshift of ribosomes translating the upstream reading frame, which would result in premature termination at out-of-phase stop codons, followed by reinitiation at the ATPase 6 start codon. Such a mechanism has been proposed to be responsible for the translation of the lysis cistron of MS2 RNA, which overlaps out of phase the coat gene on its 5' end (24). Ribosome slippage on the mRNA has been suggested as a possible mechanism accounting for the relatively frequent ($\approx 5\%$) phenotypic reversion of a frameshift mutation in yeast mtDNA (26).

The unusual organization of the ATPase 6 gene and of URFA6L discussed above may have bearing on the physiological role of the product of URFA6L by analogy with the yeast system. In mtDNA from both yeast (27) and *Aspergillus nidulans* (28), a small URF has recently been discovered upstream of the ATPase 6 gene. These URFs code for 48-amino acid-long polypeptides, exhibiting 50% sequence homology with each other, and having their first four amino acids identical to those encoded in URFA6L; significantly, mutations in the yeast URF affect the assembly or function of the ATPase (29), suggesting a crucial structural or regulatory role of the URF product in these processes. The proximity of the upstream URF and of the ATPase 6 gene in the yeast and *Aspergillus* mtDNAs, the overlapping of the putatively homologous genes in mammalian mtDNA, and the probable translation of the two proteins from the same mRNA in yeast and mammalian cells may reflect a link between expression of the two genes.

The Crossreactivity of Proteins 12 and 25 with Anti-URF1-C or Anti-URFA6L-C Antibodies. The evidence presented here indicates that the previously observed crossreactivity of components 12 and 25 with anti-URFA6L-C antibodies (11) as well as the crossreactivity detected here of the same proteins with anti-URF1-C antibodies are due to crossreactivity of the COOH-terminal peptides of the two proteins. As shown in Fig. 5, the COOH-terminal nonapeptide of the putative URF1 product has four out of nine amino acids in common and four others that are isosteric with or analogous to the amino acids found in the COOH-terminal nonapeptide of URFA6L. The antibodies against URF1-C or URFA6L-C showed a greater discriminating power between the two peptides in the immunoprecipitation experiments carried out with the whole corresponding proteins than in the reactivity tests carried out by the solid-phase radioimmunoassay with the peptides conjugated to a carrier protein. This may reflect a different accessibility of the reacting groups in the peptide sequences under the two experimental conditions. However, it is interesting that an asymmetry of crossreactivity of URF1-C and URFA6L-C when tested with anti-URF1-C and anti-URFA6L-C antibodies, with the latter antibodies exhibiting a greater discriminating power vs. the two peptides than the anti-URF1-C antibodies, was observed both in the immunoprecipitation tests and in the direct tests of crossreactivity of the peptides. The crossreactivity detected here and in previous work between URF1-C and URFA6L-C is interesting because it gives some indication of the lower limit in the

degree of homology between two peptide sequences that allows crossreactivity with antibodies raised against either of the two peptides.

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